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Intraerythrocytic Killing of Malaria Parasites

Annual Report

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Summary

The purpose of these studies is to determine the role of activated macrophages in immunity to the blood stages of malaria. This is being accomplished by comparing the activity of macrophages during lethal and non-lethal malaria infections and in malaria-resistant and non-resistant mice. We have found that peritoneal macrophages from both outbred and inbred mice infected with the non-lethal strains of *Plasmodium yoelii* produce higher levels of hydrogen peroxide than do mice infected with the lethal strain of the same parasite. In addition, spleen cells from mice infected with non-lethal *P. yoelii* have higher ADCC levels than those from a lethal infection. Further studies will determine the H_2O_2 response of spleen cells in these models, the nature of the lymphokines produced, and the ability of immune sera from each infection to enhance cytotoxicity.

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Foreword

Citations of commercial organizations and trade names in the report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)".

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Scientific Report

Research Problem

Recent evidence from our laboratory indicates that lymphokines (LK), which are produced by the spleen during rodent malaria infections stimulate normal, resident macrophages to bind and ingest and to kill intraerythrocytic malaria parasites in vitro (1, 2).

The killing of the intraerythrocytic parasites occurs across a 0.45 μ m membrane and appears to be mediated by H_2O_2 secreted by the macrophages (2). Killing is enhanced by a phagocytic stimulus to the activated macrophages (2). Human gamma-IFN (Genentech, Inc.) can activate human monocyte-derived macrophages to kill P. falciparum (3) and preliminary evidence suggests that LK obtained from malaria-infected mice also contain gamma-IFN (Ockenhouse, C. F., Spitalny, G. and Shear, H.L., in preparation).

We hypothesize that the intraerythrocytic killing of malaria parasites by macrophages is an important effector mechanism in this disease. The purpose of these studies is to determine whether this is so by determining the role of macrophage-mediated intraerythrocytic parasite killing in several in vivo models.

Background

Much evidence is accumulating to indicate that cell-mediated immune responses are very important in the response to some species of malaria (4) and may play an important part in the resistance of vaccinated mice. However, the actual mechanisms whereby cell-mediated responses protect against malaria are not known.

The overwhelming evidence supports the concept that activated macrophages are involved in controlling several infections such as Leishmania tropica (5), Rickettsia akari (6), and Trypanosome cruzi (7); that they are cytotoxic to tumor cells (8), and finally that they may be involved in vaccine-induced immunity.

We have been studying macrophage activation during rodent malaria (9) and the effects of macrophages activated with other stimulants on malaria-infected erythrocytes (1). Briefly, we have found that spleen cells of mice infected with BCG or malaria, produced factors or lymphokines (LK) which stimulated normal mouse peritoneal macrophages for enhanced phagocytosis of parasitized erythrocytes (1) and for killing of parasitized erythrocytes (2). We have also found that fresh monocytes or LK-stimulated, monocyte-derived macrophages were active in inhibiting the multiplication of P. falciparum (3). In both systems H_2O_2 seemed to be the active molecule. Killing was observed after parasitized erythrocytes bound to monocytes and was associated with an oxidative burst in the monocytes. After the interaction, the parasitized erythrocytes appeared to be degenerating and looked like the previously described "crisis" forms (10).

Our results confirm and extend several other findings. Taliaferro and Cannon (24) observed that upon acquisition of immunity in monkeys infected with malaria, some intraerythrocytic parasites appear to degenerate within the erythrocytes. This finding implied that soluble mediators might affect malarial parasites. That such mediators might be secreted by macrophages was first suggested by Allison and Clark (11). Mice treated with BCG are protected against malaria and the mechanism suggested was that parasites are killed by products of activated macrophages. Since then, other parasitocidal factors have also been shown to have an effect on intraerythrocytic malaria parasites, namely, tumor necrosis factor (12), interferon (12) and a lipopolysaccharide-induced serum factor (13).

The concept that oxygen radicals might affect malaria parasites was suggested by the observations that injections of alloran (14) and t-butyl hydroperoxide (15,16) into mice with *P. vinckei* (14, 16) or *P. yoelii* (15) markedly reduces parasitemia. These compounds generate reactive oxygen intermediates and their activity can be inhibited by iron-chelating agents such as desferrioxamine and diethylthiocarbamate. It is well known that malaria parasites are sensitive to oxidant stress. Cultures of *P. falciparum* grown in G-6-PD deficient erythrocytes are inhibited under high oxygen tension (17). In addition, dilutions of H_2O_2 as low as $10^{-3}M$ are toxic to *P. yoelii* and *P. berghei* *in vitro* and *in vivo* (18).

Early studies of Langhorne et al. (19) indicated that incubation of spleen cells from infected monkeys with parasitized erythrocytes reduced their ability to multiply. Later, Taverna et al. (20) demonstrated the killing of *P. yoelii* by cells of the monocyte-macrophage series. Data from this study also suggested that fresh blood monocytes or peritoneal cells activated by incubation with lymph node cells of immunized mice were more effective than normal peritoneal cells. Our studies show that H_2O_2 produced upon an oxidative burst in activated macrophages, is lethally damaging to *P. yoelii* and *P. falciparum*. Because these studies suggest an important protective mechanism in malaria, we feel that further studies in animal models to determine the *in vivo* relevance of this mechanism are warranted.

Approach

We will approach this by trying to answer the following questions:

1. Are there differences between lethal and non-lethal rodent malaria infections in:
 - a) the nature of the lymphokine (LK) which stimulates macrophage-mediated killing?
 - b) the ability of splenic and peritoneal macrophages from lethal and non-lethal infections to kill malaria parasites, secrete H_2O_2 and reduce nitroblue tetrazolium (NET), over the course of the infection?

c) the ability of immune serum from each infection to enhance cytotoxicity?

2. a) Are there differences between malaria-susceptible and resistant mice in their ability to produce macrophage-activating IK or in the ability of their macrophages to respond to IK, secrete H_2O_2 or reduce NBT?

b) Are there differences in the LPS non-responder mouse, C_3H/HeJ and responder mouse, C_3H/HeN , in the course of malaria infection and in the ability of their macrophages to respond to malarial IK or a second signal such as LPS and to kill parasites in vitro?

3. Is the active factor in the rodent malaria IK, gamma-IFN or another cytokine?

Results

Our first objective was to determine whether there was a difference in the ability of macrophages from mice infected with lethal and non-lethal Plasmodium yoelii infection to produce H_2O_2 , reduce NBT and mediate cytotoxicity.

1. H_2O_2 release studies.

The first series of experiments was performed on female SW mice 6-10 weeks old. Animals were infected with 10,000 infected erythrocytes. At several time points, mice were sacrificed, the peritoneal cavity rinsed, and peritoneal cells assayed for H_2O_2 release after triggering with PMA.

Our results (Fig. 1) showed that peritoneal macrophages from mice infected with the non-lethal strain begin to produce increased levels of H_2O_2 at approximately day 4 of infection. Peak amounts

of H_2O_2 are produced by approximately day 9 and then the levels decline as parasitemia declines. In contrast, macrophages obtained from mice infected with the lethal strain of *P. yoelii* did not show an increase in H_2O_2 production until day 7.

Similar results were obtained in inbred mice. In Balb/c mice which are sensitive to malaria infection, higher levels of H_2O_2 were produced by peritoneal macrophages from mice infected with non-lethal *P. yoelii* (0.98 u moles 10^6 macrophages on day 4 compared with 0.2 u moles on day 3 in the lethal infection) (Fig. 2). Experiments are in progress to determine the response of more resistant mice to lethal and non-lethal *P. yoelii*.

2. Cytotoxicity Studies.

Studies on the ability of macrophages from mice infected with lethal and non-lethal *P. yoelii* to kill intraerythrocytic parasites have yielded interesting results.

Using an antibody-dependent cell mediated cytotoxicity assay (ADCC) we determined the ability of spleen cells from SW mice infected with either lethal or non-lethal *P. yoelii* to lyse ^{51}Cr -labeled mouse erythrocytes sensitized with IgG. Figure 3 compares ADCC in mice infected for 4 days with either the lethal or the non-lethal strain of *P. yoelii*. Significantly more lysis was obtained with spleen cells from mice infected with the non-lethal variant (Shear, H. L., in preparation). This finding will be pursued by determining H_2O_2 levels in splenic macrophages during each of these infections. We have recently found that the levels of H_2O_2 produced by spleen cells during the lethal infection is much lower than levels produced by peritoneal cells. Experiments are in progress to compare splenic cells during the non-lethal infection.

We also performed experiments to determine whether parasitized erythrocytes would be lysed by ADCC. In several experiments, *P. berghei*-infected erythrocytes could not be lysed by activated peritoneal or spleen macrophages, even after presensitizing the infected cells with hyperimmune serum. In addition, *P. chabaudi*-infected erythrocytes, sensitized with either hyperimmune serum raised against *P. chabaudi*-infected erythrocyte membranes or a monoclonal antibody against a *P. chabaudi* erythrocyte membrane antigen (21) were also not lysed by activated macrophages. However, when *P. berghei*-infected erythrocytes were presensitized with rabbit anti-mouse erythrocyte IgG, they were readily lysed.

3. Studies on C3H/HeJ and C₃/O_uJ mice.

We have observed that the course of *P. yoelii* non-lethal infection is consistently slightly lower in C3H/O_uJ (LPS responders) than in C3H/HeJ (LPS non-responders) male mice. Further, the H_2O_2 levels produced by the C3H/O_uJ strain are higher in the early phase of infection in preliminary studies.

Discussion and Conclusions.

Our studies to date indicate that there are indeed differences in the macrophage response to lethal and non-lethal *P. yoelii* infections. In SW mice, peritoneal macrophages produce an earlier and higher H_2O_2 during the non-lethal infection than during the lethal infection. Studies are in progress to compare the H_2O_2 response of splenic macrophages during these infections.

The results obtained in SW mice were then confirmed in Balb/c mice which is sensitive to malaria infections. Peritoneal macrophages from mice infected with the non-lethal strain of *P. yoelii* produced higher levels of H_2O_2 than Balb/c mice infected with the lethal strain. These experiments are also being carried out in the more resistant CBA mice.

Another difference found between lethal and non-lethal infections was the ADCC (antibody-dependent cell-mediated cytotoxicity) levels of spleen cells during lethal and non-lethal infections. This finding may also reflect H_2O_2 release since H_2O_2 is thought to mediate ADCC. Experiments will be performed to compare H_2O_2 release from spleen cells during lethal and non-lethal infections.

Experiments in C3H/OuJ and C3H/HeJ showed small but consistent difference in course of infection. Preliminary studies indicate that there is also a difference in H_2O_2 release. If further experiments confirm this finding this will support the data suggested that higher H_2O_2 release is associated with a less virulent infection.

Recommendations.

During the coming year we plan to analyze the lymphokine (LK) produced during lethal and non-lethal infections and in malaria resistant and sensitive mice. We have obtained the R4 hybridoma line which produces antibody against mouse gamma-interferon and recombinant mouse gamma interferon for use as a standard. We are attempting to develop an assay for gamma IFN in LK and serum using these reagents.

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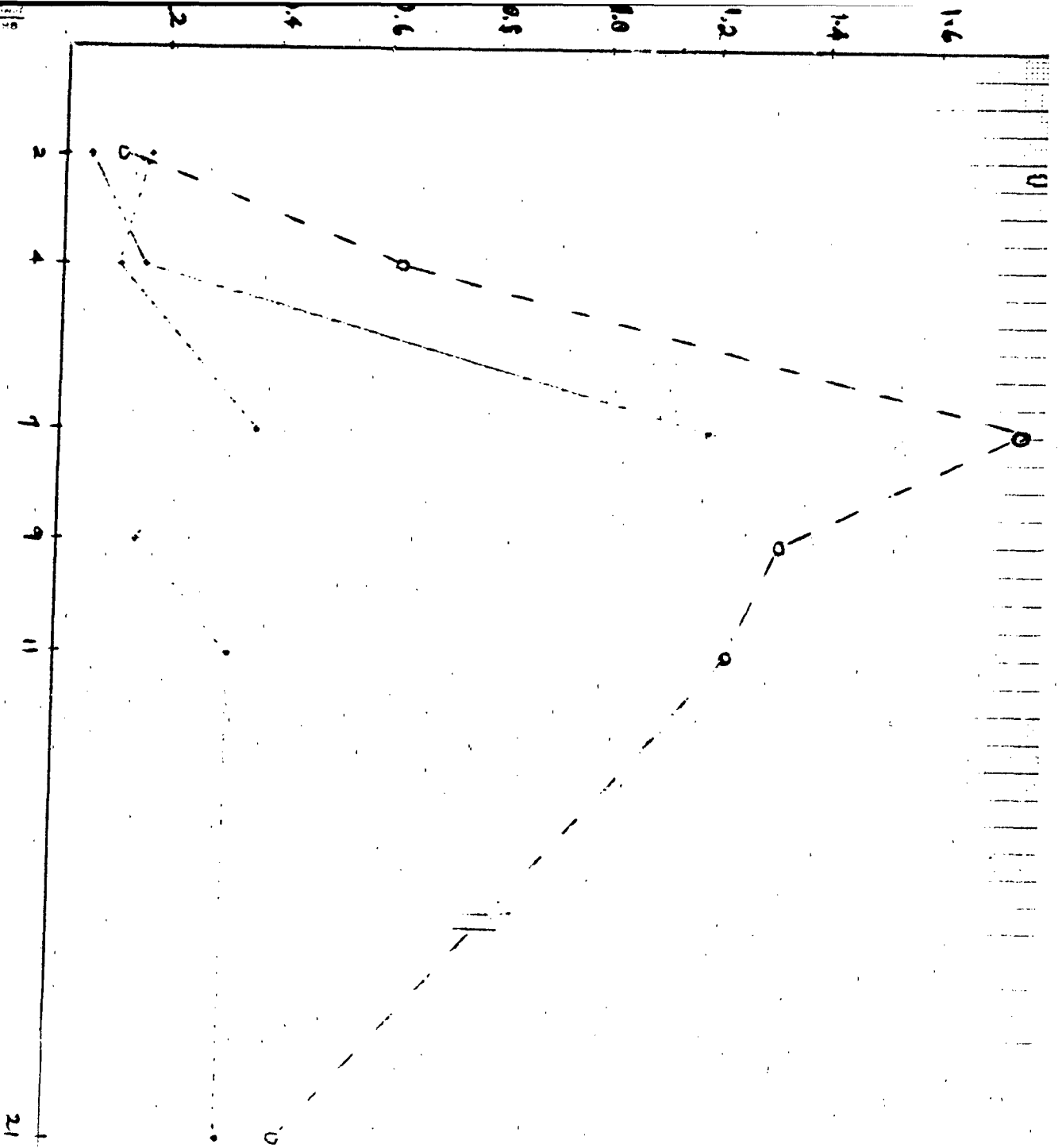
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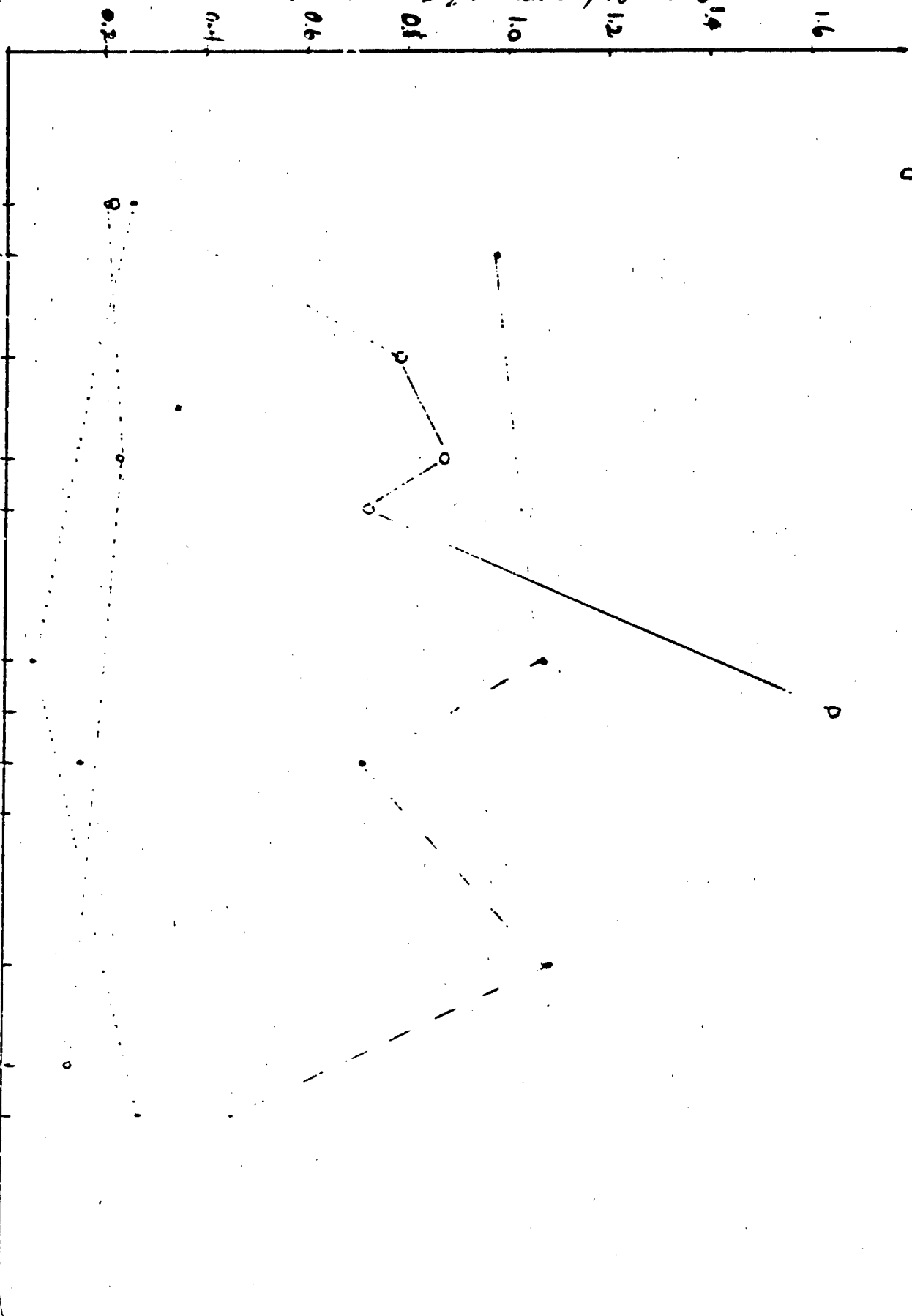
Day of infection



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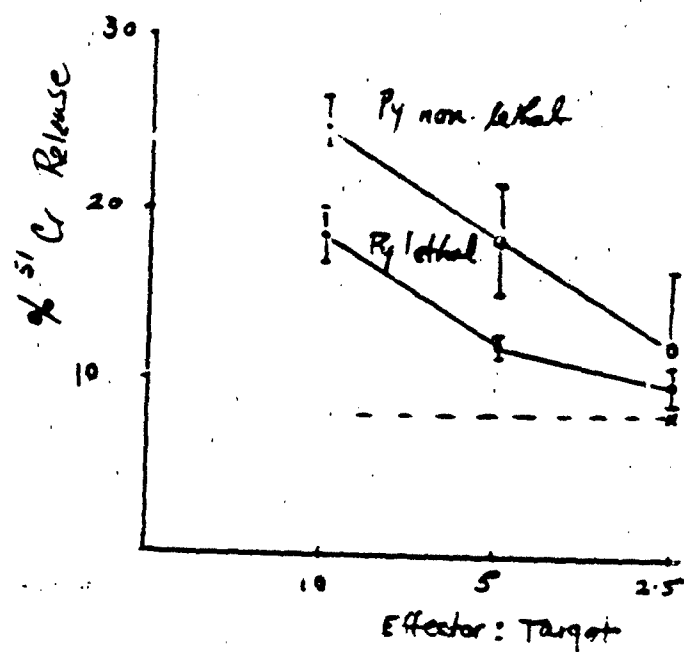
μ moles H_2O_2 released / 10^6 cells

Day of infection



D.

Figure 3



Appendix

Legends to Figures

Fig. 1. H_2O_2 released by peritoneal macrophages from SW mice during lethal and non-lethal *P. yoelii* infection. Points represent the mean of 3 mice. \circ — \circ lethal, \circ — \circ non-lethal, \circ — \circ control.

Fig. 2. H_2O_2 released by peritoneal macrophages from Balb/c mice during lethal and non-lethal *P. yoelii* infections. Points represent the mean of 3 mice. \circ — \circ lethal, \circ — \circ non-lethal, \circ — \circ control.

Fig. 3. ADCC in lethal and non-lethal *P. yoelii*. Spleen cell suspensions were assayed for ADCC against ^{51}Cr -labeled mouse E1G. Target cells were resuspended to $5 \times 10^6/ml$ and effector cells to the indicated effector:target ratio. Data represents the mean + S.D. of triplicate wells.

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